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ABSTRACT

Apigenin (Api) and *tt*-farnesol (Far) are two naturally occurring agents that affect the development of cariogenic biofilms. Fluoride (F) interferes physicochemically with caries development and also exhibits antibacterial activity. We examined whether the association of Api and Far enhance the anti-caries properties of F by acting cooperatively on the expression of virulence of *Streptococcus mutans*. The biological effects of each of the agents were greatly enhanced when used in combination with F. In general, biofilms treated with Api and/or Far in combination with F displayed less biomass and fewer insoluble glucans and iodophilic polysaccharides than did those treated with the test agents alone ($P < 0.05$). The combination of the test agents with F was highly effective in preventing caries development in rats, especially Api+Far+F, and results were comparable with those observed with chlorhexidine + F (positive control). Results from these studies showed that apigenin and *tt*-farnesol may enhance the cariostatic effectiveness of fluoride.

KEY WORDS: apigenin, *tt*-farnesol, fluoride, *S. mutans*, biofilms.

Apigenin and *tt*-Farnesol with Fluoride Effects on *S. mutans* Biofilms and Dental Caries

INTRODUCTION

Streptococcus mutans is the main pathogen responsible for the development of dental caries in humans (Tanzer *et al.*, 1985; Loesche, 1986). The organism produces glucosyltransferases (GTFs), which catalyze synthesis of glucans from dietary carbohydrates, especially sucrose. Glucans are of central importance in adhesive interactions of *S. mutans* with the tooth surface and other oral bacteria, and contribute to the formation of the matrix of dental biofilms (Yamashita *et al.*, 1993). Furthermore, *S. mutans* survives and carries out glycolysis at low pH values attained within the matrix of the biofilms, which results in demineralization of the adjacent dental enamel (Belli and Marquis, 1991; Bowen, 2002).

Recently, we have identified two potential anti-caries agents that are found in propolis, a natural beehive product (Koo *et al.*, 2002, 2003a). Apigenin (4', 5, 7-trihydroxyflavone) is a potent inhibitor of water-insoluble glucan synthesis (Koo *et al.*, 2002, 2003a); *tt*-farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) displays activities against streptococcal membranes by increasing their proton permeability and inhibits acid production by *S. mutans* within biofilms (Koo *et al.*, unpublished data). Topical application of these compounds reduced the incidence of dental caries, with minimal effects on the viability of oral flora populations *in vivo* (Koo *et al.*, 2003a).

Fluoride, in various vehicles, is the most effective anti-caries agent known (Clarkson, 2000; NIH, 2001). Nevertheless, dental caries remains a significant problem in many countries, including the United States, and continues at a high level in susceptible subpopulations, especially among economically underprivileged children (NIH, 2001). Fluoride exerts its major effect by reducing demineralization and enhancing remineralization of early caries lesions (Dawes and ten Cate, 1990). However, there is a plethora of evidence which shows that fluoride can affect the biological activities of cariogenic streptococci (Hamilton, 1990; Marquis *et al.*, 2003). For example, fluoride inhibits acid production and the production of GTFs (Bowen and Hewitt, 1974; Marquis *et al.*, 2003).

Enhancement of the protective effects of fluoride by the inclusion, in preparations, of substances which affect the virulence of cariogenic bacteria and/or enhance the antibacterial effects of fluoride offers an attractive route to reducing the prevalence of dental caries. It is generally accepted that the effectiveness of fluoride can be enhanced when it is combined with additional cariostatic agents (NIH, 2001). However, most of the compounds tested thus far are broad-spectrum antimicrobials, which suppress the resident flora (Caulfield *et al.*, 2001). In this study, we followed an alternative approach, using apigenin and *tt*-farnesol to enhance the biological effects of fluoride against *S. mutans* by simultaneously acting on the development and virulence of cariogenic biofilms.

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MATERIALS & METHODS

Test Agents

Apigenin and *tt*-farnesol were obtained from Extrasynthese Co. (Genay-Sedex, France). Sodium fluoride and chlorhexidine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). For this study, we tested 1 mM apigenin and 5 mM *tt*-farnesol, alone or in combination with sodium fluoride (250 ppm F); these concentrations were chosen based on data from our previously published and unpublished 'response to dose' and animal studies (Koo *et al.*, 2002, 2003a,b). The test agents were dissolved in 25% ethanol containing 1.25% dimethyl sulfoxide (DMSO) just prior to the initiation of the experiments. Appropriate solvent controls were always included.

Biofilm Preparation and Treatments

Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite (sHA) discs (surface area of 2.7 ± 0.2 cm², Clarkson Chromatography Products Inc., South Williamsport, PA, USA) in batch cultures for 5 days, as detailed elsewhere (Koo *et al.* 2003b; Chatfield *et al.*, 2005). The sHA discs were generated by incubation with clarified human whole saliva for 1 hr at 37°C. During the first 24 hrs, the organisms were grown undisturbed to allow for initial biofilm formation; the biofilms (24 hrs old) were then treated twice daily (one-minute exposure, at 10 a.m. and 4 p.m.) until the 5th day of the experimental period (126 hour-old biofilms) with one of the following: (i) 5 mM *tt*-farnesol (Far); (ii) 1 mM apigenin (Api); (iii) 250 ppm fluoride (F); (iv) Far + F; (v) Api + F; (vi) Api + Far + F; (vii) vehicle control (25% ethanol containing 1.25% DMSO, as negative control); or (viii) CHX + F (as positive control). Each biofilm was exposed to the respective treatment a total of 8 times. Biofilm assays were performed in quadruplicate in at least three different experiments.

Biofilm Analyses

At the end of the experimental period, the biofilms were gently washed in physiological saline (0.89% NaCl, w/v) for removal of loosely adherent material. We used one set of biofilms for *in situ* pH measurements by placing the tip of a Beetrode pH electrode (World Precision Instruments, New Haven, CT, USA) into the matrix of the biofilms; a series of pH readings was recorded from 10 different sites (Li and Burne, 2001). Additional sets of biofilms

were analyzed for: (i) biomass (dry weight), (ii) number of viable cells, (iii) total protein (ninhydrin assay; Moore and Stein, 1954), and (iv) polysaccharide composition (soluble and insoluble glucans, and intracellular iodophilic polysaccharides), by means of colorimetric (Dubois *et al.*, 1956; Di Persio *et al.*, 1974) and scintillation counting (Koo *et al.*, 2003b) methods.

Animal Studies

The animal experiment was performed according to methods described previously (Koo *et al.*, 2003a). At weaning, pups aged 21 days were infected by *S. mutans* UA159, and randomly placed into 8 groups of 12 animals, and their teeth were treated topically by means of a camel's hair brush twice daily, as follows: (1) 5 mM Far, (2) 1 mM Api, (3) 250 ppm F, (4) Far + F, (5) Api + F, (6) Api + Far + F, (7) vehicle control (25% ethanol containing 1.25% DMSO, as negative control), or (8) CHX + F (as positive control). Each group of 12 animals was provided with National Institutes of Health diet 2000 (which contains 56% sucrose) and 5% sucrose water *ad libitum*. The experiment proceeded for 5 wks, at the end of which the animals were killed by CO₂ asphyxiation. The microbiological assessment and caries evaluation were carried out by previously described methods (Koo *et al.*, 2003a). This study was reviewed and approved by the University of Rochester Committee on Animal Resources.

Statistical Analyses

For the *in vitro* studies, the data were analyzed by ANOVA, and we used the F-test to test any difference between and among the groups. When significant differences were detected, pairwise comparison was made among all the groups by Tukey's method to adjust for multiple comparisons. For the animal studies, smooth-surface and sulcal caries scores were expressed as proportions of their maximum possible values (124 and 56). The data were subjected to ANOVA in the Tukey-Kramer Honest Standard Deviation (HSD) test for all pairs. Statistical software JMP version 3.1 (SAS Institute, Cary, NC, USA) was used to perform the analyses. The level of significance was set at 5% for both studies.

RESULTS

All the test agents, with the exception of fluoride alone, diminished the further accumulation of *S. mutans* biofilms compared with the vehicle control ($P < 0.05$) (Table 1). The

Table 1. *Streptococcus mutans* UA159 Biofilm Composition and Acidogenicity after Treatments

Treatments ^a	Vehicle Control	250 ppm Fluoride (F)	5 mM #Farnesol (Far)	1 mM Apigenin (Api)	Far + F	Api + F	Api + Far + F
Dry-weight (mg)	8.1 (0.4)	7.2 (0.3)	6.4 (0.4)	6.0 (0.2)	5.4 (0.3)	4.8 (0.2)	4.0 (0.3)
Total protein (mg)	2.1 (0.2)	1.7 (0.3)	1.5 (0.2)	1.4 (0.1)	1.2 (0.3)	1.0 (0.1)	0.9 (0.0)
cfu (x 10 ⁸)	1.4 (0.2)	1.5 (0.4)	0.8 (0.2)	1.0 (0.5)	0.8 (0.2)	1.1 (0.1)	0.7 (0.3)
Insoluble glucans (mg)	2.4 (0.2)	2.0 (0.4)	1.7 (0.2)	1.6 (0.1)	1.4 (0.5)	1.2 (0.1)	1.0 (0.3)
Soluble glucans (mg)	0.62 (0.06)	0.58 (0.05)	0.58 (0.03)	0.57 (0.02)	0.56 (0.03)	0.51 (0.04)	0.45 (0.01)
IPS (mg)	0.58 (0.05)	0.28 (0.04)	0.37 (0.03)	0.46 (0.05)	0.18 (0.02)	0.24 (0.00)	0.13 (0.01)
<i>In situ</i> pH	4.3 (0.3)	4.8 (0.2)	4.6 (0.1)	4.4 (0.4)	5.2 (0.2)	4.8 (0.2)	5.4 (0.3)

Values (SD, N = 12) joined by underlines or connected by lines are not significantly different from each other ($P > 0.05$, ANOVA, comparison for all pairs using Tukey test).

^a Twice daily with one-minute exposure for each treatment; chlorhexidine + fluoride (CHX+F) treatments halted further accumulation of biofilms.

Table 2. Effects of Treatments on Microbial Population in the Animals' Plaque

Treatments	Vehicle Control	5 mM <i>tt</i> -Farnesol (Far)	1 mM Apigenin (Api)	250 ppmF (F)	Api + F	Far + F	Api + Far + F	CHX + F
Total counts (cfu x 10 ⁶ /mL)	2.31 (0.71)	2.21 (0.97)	2.57 (1.01)	2.31 (2.23)	2.54 (1.95)	2.62 (0.53)	2.28 (1.48)	1.50 (0.32)
<i>S. mutans</i> counts (cfu x 10 ⁶ /mL)	0.51 (0.17)	0.45 (0.06)	0.49 (0.11)	0.48 (0.52)	0.57 (0.08)	0.60 (0.14)	0.58 (0.21)	0.32 (0.08)
% of <i>S. mutans</i>	22.0 (0.9)	20.4 (4.3)	19.1 (2.3)	21.0 (1.2)	22.4 (6.1)	22.9 (1.1)	25.4 (4.4)	21.3 (0.5)

Means (SD, N = 12).

Values joined by underlines or connected by lines are not significantly different from each other ($P < 0.05$; ANOVA, comparison for all pairs by Tukey-Kramer HSD).

combinations of Api + F and Api + Far + F were the most effective treatments and resulted in 40.7% and 50.6% less biomass (dry-weight), respectively, than did the vehicle control treatment; concentration of protein was affected similarly. None of the test agents displayed bactericidal activity.

The total amount of polysaccharides in the biofilms was affected by treatments with the test agents. The amount of insoluble glucans in the biofilms treated with Api and Far, alone or in combinations, was significantly less than in those treated with vehicle control ($P < 0.05$, Table 1); the combinations Api + F and Api + Far + F were more effective than each of the test agents alone ($P < 0.05$). The amount of water-soluble glucans in the biofilms was unaffected by the test agents, except those treated with the combination Api + Far + F. In contrast, the amount of iodophilic polysaccharide was drastically reduced by F, alone or in combinations (Api + F, Far + F, and Api + Far + F) (51.7 to 77.6% reduction).

The acidogenicity of the treated biofilms was reduced only by Far + F and Api + Far + F. The pH values measured in the biofilms that were treated with the combinations were 0.9 to 1.1 units higher than those treated with vehicle control 16 hrs after the final treatment ($P < 0.05$, Table 1).

Our positive control CHX + F displayed bactericidal activity against the early-formed *S. mutans* biofilms, and halted

the further accumulation of the biofilms (data not shown).

In the animal experiment, weight gains were not significantly different among the control and test agent groups ($P > 0.05$). The percentage of *S. mutans* UA159 recovered from the jaws of the rats was calculated from total cultivable flora and the *S. mutans* population. The percentage of *S. mutans* in the animals' plaque was similar among all groups, and ranged from 19.1% to 25.4% (Table 2). However, the group treated with CHX + F displayed significantly lower counts of both total and *S. mutans* populations compared with the control group ($P < 0.05$).

The incidence of smooth-surface caries was reduced in all treatment groups compared with the control (30 to 65% reduction, $P < 0.05$; Table 3), with the exception of the group treated with 5 mM *tt*-farnesol. The smooth-surface caries severity scores were also significantly lower in all groups treated with the test agents (Table 3). However, only the groups treated with Api + Far + F and CHX + F had fewer severe lesions than groups treated with the test agents (Api or Far) or fluoride alone (at Ds level only, $P < 0.05$). Furthermore, the animals treated with Api + Far + F were the only group free of Dx scores.

The incidence of sulcal-surface caries was significantly lower in the groups treated with F, either alone or in

Table 3. Effects of Treatments on Development (smooth surface and severity) of Dental Caries in Rats

Treatments	Vehicle Control	5 mM <i>tt</i> -Farnesol (Far)	1 mM Apigenin (Api)	250 ppmF (F)	Far + F	Api + F	Api + Far + F	CHX + F
Smooth-surface caries	40.9 (11.7)	28.8 (10.0)	27.7 (9.4)	21.2 (8.0)	17.3 (9.1)	14.8 (5.5)	14.5 (5.3)	15.0 (9.1)
Smooth-surface severity ^a								
(Ds)	11.9 (6.2)	4.3 (3.1)	5.6 (4.7)	2.1 (1.2)	1.5 (2.0)	1.2 (1.4)	0.4 (0.7)	0.8 (1.1)
(Dm)	5.8 (3.1)	2.0 (1.4)	1.8 (2.5)	0.7 (0.6)	0.5 (0.7)	0.7 (1.0)	0.1 (0.2)	0.5 (1.0)
(Dx)	3.1 (2.1)	0.6 (0.8)	0.4 (0.7)	0.4 (0.1)	0.1 (0.3)	0.5 (0.8)	n.d.	0.2 (0.6)

Means (SD, N = 12).

Values joined by underlines or connected by lines are not significantly different from each other ($P < 0.05$; ANOVA, comparison for all pairs by Tukey-Kramer HSD).

^a Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, whole dentin affected.

Table 4. Effects of Treatments on Development (sulcal-surface caries and severity) of Dental Caries in Rats

Treatments	Vehicle Control	5 mM <i>tt</i> -Farnesol (Far)	1 mM Apigenin (Api)	250 ppmF (F)	Far + F	Api + F	Api + Far + F	CHX + F
Sulcal-surface caries	47.8 (4.3)	44.0 (3.3)	<u>41.7 (4.8)</u>	32.3 (4.0)	34.8 (5.1)	31.6 (5.6)	33.5 (4.1)	32.4 (6.8)
Sulcal-surface severity ^a								
(Ds)	39.3 (3.7)	36.2 (3.5)	<u>35.0 (4.0)</u>	29.2 (4.3)	27.7 (5.9)	25.9 (6.1)	26.9 (5.6)	24.3 (6.5)
(Dm)	<u>25.0 (5.8)</u>	<u>19.4 (5.0)</u>	<u>19.0 (4.8)</u>	16.2 (4.6)	<u>12.8 (6.9)</u>	11.8 (5.9)	11.0 (6.1)	10.3 (6.2)
(Dx)	<u>5.8 (2.8)</u>	<u>2.1 (1.5)</u>	<u>1.9 (1.3)</u>	<u>1.2 (1.4)</u>	0.6 (1.5)	0.6 (0.8)	0.4 (1.2)	0.4 (0.8)

Means (SD, N = 12).

Values joined by underlines or connected by lines are not significantly different from each other ($P < 0.05$; ANOVA, comparison for all pairs by Tukey-Kramer HSD).

^a Ds, dentin exposed; Dm, 3/4 of the dentin affected; Dx, whole dentin affected.

combinations, than in the control group ($P < 0.05$; Table 4). The severity of sulcal lesions followed a pattern similar to that of sulcal-surface caries scores; animals treated with fluoride (alone or in combinations) exhibited lower scores at Ds level compared with the control group ($P < 0.05$). However, only the combinations Api + F, Api + Far + F, and CHX + F were effective in reducing the sulcal-surface severity at Dm and Dx levels compared with the control group ($P < 0.05$).

DISCUSSION

Analysis of the data shows that the combination of Api and Far with F significantly reduced the virulence of *S. mutans*, which resulted in enhanced cariostatic properties without significant effect on the viability of the oral flora population (both total and *S. mutans*) in plaque from the animals. Among all the different combinations of the test agents, Api + Far + F displayed the maximum therapeutic effect *in vivo*, and its potency was comparable with that of our positive control, CHX + F. It appears that Api and Far, acting in concert with F on the virulence of *S. mutans* involved in the formation of cariogenic biofilm communities, caused significant changes in the insoluble glucan and intracellular polysaccharide (IPS) content of the biofilms.

The putative pathways by which Api, Far, and F affect the cariogenicity of *S. mutans* may involve several routes. We propose at least three: (1) inhibition of glucan synthesis, (2) disruption of acid production and acid tolerance, and (3) affecting IPS accumulation. Apigenin is a potent inhibitor of GTFs B and C, either in solution or adsorbed onto a sHA surface, and also affects the expression of *gtfB* and *gtfC* genes (Koo *et al.*, 2003a; unpublished data). These enzymes are responsible for the synthesis of insoluble glucans, which are critical in the expression of virulence in the pathogenesis of dental caries (Yamashita *et al.*, 1993). In contrast, F and Far affect the synthesis of exopolysaccharides without direct effects on GTF activity (Bowen and Hewitt, 1974; Marquis *et al.*, 2003). Enzyme secretion by bacterial cells is generally coupled to Δp , the proton-motive force, across the cell membrane. Because F and Far act to diminish Δp by increasing proton permeability and discharge of ΔpH across the cell membrane (Marquis *et al.*, 2003), it is probable that they will affect the

secretion of GTFs and thereby diminish the synthesis of glucans. Thus, Api and Far, acting cooperatively with fluoride, could reduce the amount of glucan in the biofilm. Analysis of our data supports this hypothesis, because it is evident that the quantity of extracellular insoluble glucans in *S. mutans* biofilms was reduced in the presence of Api + Far + F.

Bacteria such as the mutans streptococci can carry out glycolysis at low pH values, even though glycolytic enzymes are not acid-tolerant, because they maintain ΔpH across the cell membrane, with the interior more alkaline than the exterior. During glycolysis, protons are moved out of the cell through the proton-translocating membrane F-ATPase. Fluoride short-circuits this flow through the diffusion, into cells, of HF, which acidifies the cytoplasm and inhibits intracellular enzymes (Marquis *et al.*, 2003). In contrast, *tt*-farnesol changes the permeability and fluidity of the cell membrane by its lipophilic properties, which favor localization in the membrane (Koo *et al.*, 2002; Ramage *et al.*, 2002; Inoue *et al.*, 2004). Apigenin is without effect on proton-permeability of the membrane of *S. mutans*. However, Api exhibited moderate inhibitory effects on the activity of F-ATPase (25% inhibition), which could affect the acid-tolerance of *S. mutans*. Cytoplasmic acidification caused by these agents could disrupt the glycolytic acid production and the formation of intracellular iodophilic polysaccharides (IPS), a glycogen-like storage polymer (Hamilton, 1990). The IPS provide *S. mutans* with an endogenous source of carbohydrate which can be metabolized when exogenous fermentable substrate has been depleted in the oral cavity (Hamilton, 1976); as a result, IPS can promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids, with a concomitant lower fasting pH in the matrix of the plaque (Tanzer *et al.*, 1976). The importance of IPS to *S. mutans* virulence supports previous reports in the literature that describe an association of these storage polysaccharides and dental caries in both animals and humans (Loesche and Henry, 1967; Tanzer *et al.*, 1976; Spatafora *et al.*, 1995). It is noteworthy that the biofilms with the least amount of IPS had the highest pH values, especially those treated with Api + Far + F (Table 1). It is apparent that, by disrupting the accumulation of IPS, the combination of agents is reducing the acidogenicity of the biofilms, thereby

affecting the development of dental caries in rats. Whether these agents can affect the synthesis of extracellular or intracellular polysaccharides, or acid production by other cariogenic organisms, awaits further evaluation.

The combination of these novel agents with fluoride may represent a potentially useful alternative approach to the current chemotherapeutic strategies to prevent this ubiquitous disease, by reducing the expression of virulence of *S. mutans* without necessarily suppressing the resident oral flora. Although details of the toxicology of Api and Far were not investigated here, we did not observe any adverse reactions in our animal study. We are currently investigating the molecular mechanism(s) of action of these agents in combination.

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